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THERAPEUTIC APPROACHES TO THE TREATMENT OF BOTULISM

Final Report

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FOREWORD

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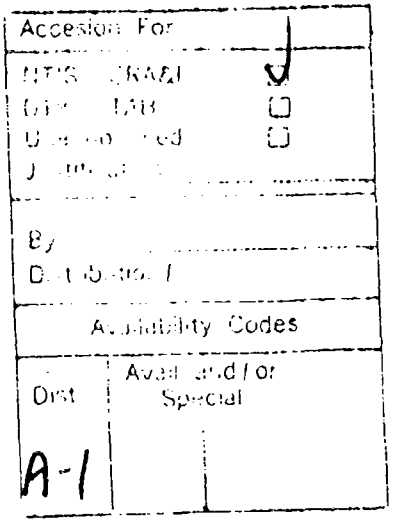

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Research has been conducted in four areas that pertain to the development of antagonists of botulinum neurotoxin, as follows:

1.) Studies on monoclonal antibodies indicate that they are useful research tools, but they appear to have little therapeutic potential.

2.) Research on aminopyridines and their analogues shows that they possess anti-botulinum activity, but only of a narrow utility.

3.) Experiments with dendrotoxin have resulted in the provocative finding that this agent does not antagonize any clostridial toxin, nor does it reverse the effects of low calcium or high magnesium.

4.) Initial studies on rubidium flux indicate that this may represent a rational approach for finding clostridial toxin antagonists.

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1. Statement of Problem

Pharmacological methods are being sought to prevent or reverse the effects of botulinum neurotoxin. Emphasis has been placed on the study of aminopyridines and related drugs that affect potassium channels.

2. Background

Botulinum neurotoxin exists in seven serotypes that are designated A,B,C,D,E,F and G. The various forms are relatively or absolutely distinct immunologically, but they share a similar macrostructure and perhaps a similar mechanism of action. Of the seven serotypes, three account for most cases of human botulism (A,B and E) and one accounts for most cases of animal botulism (C). The present study deals with types A,B,C and E botulinum neurotoxin.

Each of the botulinum neurotoxins is synthesized as a single chain polypeptide with a molecular weight of approximately 150,000 (For a review on structure, see Sakaguchi, 1983; for a review on mechanism of action, see Simpson, 1986a). When this precursor is exposed to proteolytic cleavage, it is converted to a fully active molecule in which a heavy chain (Mr ~ 100,000) is linked by a disulfide bond to a light chain (Mr ~ 50,000). The heavy chain is thought to be responsible for binding and internalization of the toxin. The light chain is assumed to be an enzyme that acts intracellularly to block acetylcholine release.

There are many similarities between botulinum neurotoxin and tetanus toxin (DasGupta and Sugiyama, 1977; Habermann and Dreyer, 1986; Simpson, 1986a). The latter substance is synthesized and activated almost identically to botulinum neurotoxin. Furthermore, tetanus toxin has a two chain structure that appears to have the same functional domains as botulinum neurotoxin. The most compelling evidence for similarity comes from a recent study on homology. The gene for tetanus toxin has been sequenced, and from this an amino acid sequence has been deduced (Eisel et al., 1986; Fairweather and Lyness, 1986). A comparison of the complete primary structure of tetanus toxin with the partial primary structure of several botulinum neurotoxins reveals substantial homology (Eisel et al., 1986). These various lines of evidence suggest that botulinum neurotoxin and tetanus toxin may be evolutionary descendants of the same ancestral parent.

The idea of commonality in origin, structure and activity is appealing, but there is at least one observation that may be a challenge to the hypothesis. A number of authors have reported that aminopyridines such as 4-aminopyridine (4-AP) and 3,4-diaminopyridine have markedly different activities in antagonizing clostridial neurotoxins. The drugs are potent antagonists of type A botulinum neurotoxin, but they are only weak antagonists of the other neurotoxins (Dreyer and Schmitt, 1981; Habermann et al., 1980; Kauffman et al., 1985; Lewis, 1981; Lundh et

al., 1977; Lundh and Thesleff, 1977; Sellin et al., 1983; Simpson, 1978; 1986b). The data on aminopyridines have prompted questions about commonalities among the toxins.

An accepted approach for determining site and mechanism of action of pharmacological agents is to compare the magnitude of evoked responses in the presence of antagonists. The present study compares the neuromuscular blocking actions of botulinum neurotoxins types A,B,C and E and tetanus toxin. Comparisons have been carried out in the presence or absence of antagonists that inhibit internalization of toxins or inhibit intracellular toxicity. The results have been compared with previously published data on antagonism of binding. The collective findings are used to deduce the extent of relatedness among the clostridial neurotoxins.

In a related vein, recent work by the Principal Investigator and by others has drawn attention to a number of anomalies. Firstly, 4-AP and its analogs are potassium channel blockers, and this secondarily promotes calcium influx and acetylcholine efflux. An action like this would be predicted to antagonize botulinum neurotoxin. However, 4-AP and its analogs are strong antagonists of only serotype A.

A second anomaly pertains to dendrotoxin. This substance is also a potassium channel blocker, and thus it too would be expected to antagonize toxins that block exocytosis. Indeed, when tested against beta-bungarotoxin

it did delay onset of paralysis (see below). But when tested against other phospholipase neurotoxins (e.g., crotoxin), it did not afford protection.

Finally, there is a "crossed anomaly". 4-AP can protect against at least one of the serotypes of botulinum neurotoxin, but it does not protect against any of the phospholipase neurotoxins. Conversely, dendrotoxin can protect against beta-bungarotoxin, but it does not protect against any of the clostridial toxins (again, this report).

The present study provides data from a large scale screening process in which various putative antagonists were tested against various neuromuscular blocking agents. These data are a prelude to trying to unravel the basis for the numerous apparent anomalies.

3. Materials and Methods

Tissue preparations. Phrenic nerve-hemidiaphragms were excised from mice (20-30 g; female, Swiss-Webster, Ace Animal, Inc.) and suspended in a 20 ml tissue bath containing a physiological solution that was bubbled with 95% O_2 and 5% CO_2. Unless otherwise indicated, the physiological solution had the following composition (millimolar): NaCl, 137; KCl, 5; $CaCl_2$, 1.8; $MgSO_4$, 1.0; $NaHCO_3$, 24; NaH_2PO_4, 1.0; and glucose, 11. The solution was supplemented with gelatin (0.02%) to diminish nonspecific inactivation of toxins.

Phrenic nerves were stimulated supramaximally with bipolar electrodes. Parameters of nerve stimulation were 0.2 Hz square waves of 0.1 to 0.3 msec duration. Muscle twitch was recorded by a force-displacement transducer connected to a physiological recorder. Toxin-induced paralysis of neuromuscular transmission was measured as a 90% reduction in muscle twitch amplitude evoked by nerve stimulation.

Toxins and drugs. Types A,B,C and E botulinum neurotoxin were purchased from Wako Chemicals (Dallas, TX). Tetanus toxin was purchased from Calbiochem (San Diego, CA) and cholera toxin was purchased from Sigma Chemical Co. (St. Louis, MO). Ammonium chloride was obtained from Fisher Scientific Co. (Fair Lawn, NJ), methylamine hydrochloride was obtained from Aldrich Chemical Co. (Milwaukee, WI), and all other drugs were purchased from Sigma Chemical Co.

Assay for neurotoxins. . . various neurotoxins were not purified to homogeneity. The removal of auxiliary proteins and in some cases nucleic acid renders the neurotoxins unstable. Therefore, experiments were done with the stable neurotoxin-auxiliary protein complexes.

Each of the four botulinum neurotoxins and tetanus toxin was bioassayed on the phrenic nerve-hemidiaphragm. For each toxin, a concentration was chosen that produced paralysis within 100 to 120 minutes and this was the concentration used in all experiments. Equiactive concentrations of toxin were re-bioassayed on tissues in the

presence of drugs thought to be antagonists. Drugs that actually behaved as antagonists produced an apparent decrease in toxicity, i.e., produced an increase in the amount of time necessary for onset of toxin-induced paralysis. Results are presented in terms of absolute changes in paralysis times.

4. Results

A. Aminopyridines and related antagonists

Ammonium chloride and methylamine hydrochloride. The paradigm used with ammonium chloride and methylamine hydrochloride was the same as that used with all potential antagonists. It consisted of a sequence of three experiments, as follows: i.) a determination of the concentration range within which a suspected antagonist can be applied to the isolated neuromuscular junction, ii.) a determination of whether and at what concentration(s) the antagonist alters the activity of botulinum neurotoxin and tetanus toxin, and iii.) a determination of the time(s) during toxin-induced paralysis when the antagonist must be added to exert its protective effect.

Previous studies have shown (Simpson, 1983), and preliminary experiments here have confirmed, that high concentrations of ammonium chloride and methylamine hydrochloride depress neuromuscular transmission. The highest concentration of ammonium chloride that can be used

is about 8 mM; that of methylamine hydrochloride, about 20 mM.

Various concentrations of drug were added to tissues simultaneously with toxin, and paralysis times were monitored. It was determined that 8 mM ammonium chloride and 10 mM methylamine hydrochloride produced maximal effects. When higher concentrations of ammonium chloride were tested, the deleterious effects of the drug added to those of the clostridial toxins. When higher concentrations of methylamine hydrochloride were tested, the drug continued to exert a strongly protective effect, but no greater than that seen at 10 mM. As already reported, both antagonists must be added to tissues simultaneously with, or only shortly after, a clostridial toxin (Simpson, 1983). If they are added after toxin-induced blockade has begun to emerge, they no longer exert a protective effect.

Antagonism by calcium. Paralysis times of tissues were measured in the presence of equiactive concentrations of neurotoxin and varying concentrations of calcium (1, 2, 4, 8, 16 and 32 mM). The highest concentration of calcium (32 mM), either with or without osmotic and/or ionic compensation, depressed transmission, but the effect was not total and it was reversible.

The toxins appeared to segregate into two groups, based on their interaction with calcium. Botulinum neurotoxin type A was significantly antagonized by increasing concentrations of calcium, from 1 mM to 16 mM. When the

concentration of calcium was increased to 32 mM, the antagonistic effect was reduced. A qualitatively similar profile was obtained with botulinum neurotoxin types B, C and E, as well as tetanus toxin, but the magnitude of effect was less.

Antagonism by 3,4-diaminopyridine and by guanidine. The dose-response characteristics of 3,4-diaminopyridine action on the mouse phrenic nerve hemidiaphragm have been reported (Simpson, 1986b). The highest concentration that is practical for use is approximately 0.2 mM. On the other hand, guanidine can be used at concentrations as high as 8 mM.

Several investigators have demonstrated that botulinum neurotoxin is antagonized by aminopyridines and by guanidine (see above). Therefore, varying concentrations of 3,4-diaminopyridine and guanidine were tested for their abilities to antagonize serotype A. The results indicated that 0.1 mM 3,4-diaminopyridine and 3.0 mM guanidine exerted a maximal effect. These concentrations were then tested against other serotypes of botulinum neurotoxin and against tetanus toxin.

3,4-Diaminopyridine and guanidine exerted a somewhat selective effect. They dramatically antagonized botulinum neurotoxin type A; paralysis times were increased more than 2-fold, giving an apparent decrease in toxicity of more than 90%. By contrast, the drugs had little or no effect when tested against botulinum neurotoxin types B, C and E or

against tetanus toxin. Paralysis times increased modestly or not at all.

It has previously been reported that the ability of aminopyridines to exert a protective effect against botulinum neurotoxin type A is time-dependent (Simpson, 1986b). Experiments were done to determine whether there was a similar time-dependency with guanidine. The data showed that when the drug was added to tissues prior to, simultaneously with, or 30 minutes after toxin, its protective effect was maximal. When the drug was added at later times, its protective effect was diminished. The drug had no observable effect on tissues that were fully paralyzed.

The interaction between calcium and 3,4-diaminopyridine or guanidine. Two types of experiments were done. In the first, low levels of calcium (1.0 mM) were used with botulinum neurotoxin type A. The purpose of the experiment was to determine whether a decrease in the calcium concentration would alter the antagonistic activity of 3,4-diaminopyridine or guanidine. In the second experiment, elevated levels of calcium (8.0 mM) were used with botulinum neurotoxin types B, C and E and tetanus toxin. The purpose was to determine whether increased amounts of calcium would allow 3,4-diaminopyridine and guanidine to express an antagonistic effect.

In the initial experiment, the results were striking. Reducing the levels of ambient calcium from 1.8 mM to 1.0 mM

greatly diminished the antagonism due to 3,4-diaminopyridine (0.1 mM) and guanidine (3 mM;). Increasing the concentration of the antagonists twofold did not restore the protective effect. In the second experiment, the results were less striking. Increasing the concentration of calcium from 1.8 mM to 8.0 mM did little to enhance the activity of 3,4-diaminopyridine and guanidine. Indeed, drug-induced antagonism of botulinum neurotoxin type A at a calcium level of 1.8 mM was greater than drug-induced antagonism of any of the other neurotoxins at a calcium level of 8.0 mM.

Theophylline, forskolin, isobutylmethylxanthine, and cholera toxin. Four drugs known to elevate tissue levels of C-AMP were tested for their abilities to antagonize clostridial neurotoxins. One of these drugs (theophylline) has previously been examined as an antagonist of one botulinum neurotoxin serotype (A; Howard et al., 1976).

The addition of theophylline to isolated neuromuscular preparations produced concentration-dependent increases in nerve-evoked muscle twitch (0.5 mM to 4.0 mM). The effect was maximal at 2.0 mM ($n = 48$; $31 \pm 4\%$; $p < 0.01$). However, this enhanced response waned with time. Even in the absence of toxins, the increase in twitch amplitude returned to control levels within 60 to 100 minutes.

When tissues were pretreated with theophylline (2 mM; 15 minutes) prior to the addition of neurotoxins, the drug produced an effect that was not universal. Theophylline significantly antagonized botulinum neurotoxin type A (n =

12; control = 118 ± 9 minutes; experimental = 137 ± 12; $p < 0.05$), but it did not antagonize the other botulinum neurotoxins or tetanus toxin to an extent that attained statistical significance.

Equivalent experiments were done with forskolin (1.0 to 100 M), isobutylmethylxanthine (0.1 to 5 mM) and cholera toxin (10^{-10} to 10^{-7} M). Forskolin did not enhance muscle twitch, nor did it antagonize any of the clostridial neurotoxins. Isobutylmethylxanthine enhanced muscle twitch (e.g., 1 mM; n = 27; 43 ± 2%; $p < 0.01$), but the enhanced twitch waned with time. When tissues were pretreated with the drug (1 mM; 15 minutes), there was no significant antagonism of toxin-induced neuromuscular blockade.

At concentrations of 10^{-10} to 10^{-7} M, cholera toxin produces characteristic morphological changes and simultaneous increases in tissue levels of C-AMP in a cell line that has been used to study various toxins (Zepeda et al., submitted for publication). These concentrations did not enhance neuromuscular transmission nor did they antagonize clostridial neurotoxins. This result was obtained when cholera toxin was added 1, 2, 3 or 4 hours prior to a clostridial toxin.

B. Aminopyridines and PLA2 Neurotoxins.

Three snake neurotoxins were tested: beta-bungarotoxin (obtained commercially), crotoxin (isolated in the Principal Investigator's lab), and notexin (obtained from a

collaborator). Each was titrated on the mouse phrenic nerve-hemidiaphragm preparation to produce an eventual paralysis time of 100 to 120 minutes.

Two groups of tissues were then exposed to equiactive concentrations of toxin. A control group was treated only with toxin; an experimental group was titrated with 4-AP to produce at least a 50% enhancement in muscle twitch (conc.~ 10^{-4} M). The data (Table 1) show that 4-AP was not an effective antagonist against any of the PLA2 neurotoxins.

C. Dendrotoxin and Clostridial Neurotoxins.

Similarly to the previous series of experiments, the clostridial neurotoxins were added to tissues at concentrations that produced paralysis in 100 to 120 minutes. Types A, B, and E neurotoxin were tested. Type E was activated with trypsin before addition to neuromuscular preparations. As an internal control, experiments were also done with dendrotoxin and beta-bungarotoxin.

As expected, dendrotoxin (Table 2) was an antagonist of beta-bungarotoxin. When tissues (n=5) were exposed only to the PLA2 neurotoxin, the eventual paralysis times were 117±14 min. (Table 2). This was in marked contrast to the findings with the clostridial neurotoxins. In the latter case, dendrotoxin never afforded protection.

D. Potassium channel blockers and magnesium.

Magnesium is an effective neuromuscular blocking agent whose mechanism of action is well known: it is a competitive antagonist of calcium. When calcium levels in physiological solution are lowered (1.0 mM), increases in the levels of magnesium (10 -15 mM) will paralyze transmission.

Individual tissues were paralyzed by lowering calcium and increasing magnesium. Tissues were then treated with 4-AP or with dendrotoxin. The results (Table 3) showed an interesting outcome. 4-AP was able to completely overcome Mg-induced blockade, but dendrotoxin was almost completely ineffective.

Although additional experiments are needed, the results with magnesium suggest that there may be a "mislabeling" in the literature. Although dendrotoxin does block potassium channels and does enhance calcium flux, this is not a major action. It is not, for example, an action that is capable of overcoming magnesium-induced block. Most probably, there is some other action that accounts for the ability of dendrotoxin to antagonize beta-bungarotoxin. One possibility is competition for a common binding site.

The same may be true for 4-AP and its analogues. It purportedly antagonizes type A botulinum toxin by virtue of being a potassium channel blocker. However, this is an hypothesis that has not been proved, and other explanations are possible.

TABLE 1

The Interaction Between 4-AP and PLA2 Neurotoxins

Toxin	Paralysis Time [1]	
	Control [2]	4-AP[2]
Beta-Bungarotoxin	109±8	111±12
Crotoxin	101±9	108±7
Notexin	117±13	105±4

[1] Minutes (Mean ± SEM)

[2] Group N=5 or more

TABLE 2

The Interaction Between Dendrotoxin and Presynaptic Neurotoxins

Toxin	Paralysis Time [1]	
	Control [2]	Dendrotoxin [2]
Beta-Bungarotoxin	117A13	161A14 [3]
Botulinum Toxin-A	121±8	116±3
Botulinum Toxin-B	107±5	116±13
Botulinum Toxin-E	112±9	120±14

[1] Minutes

[2] Group N=5 or more

[3] Significantly different from control ($p<0.01$)

TABLE 3

The Interaction Between 4-AP or Dendrotoxin and Magnesium

Time [1]	Treatment [2]		
	None	4-AP	Dendrotoxin
1	0	0	0
2	0	1	0
4	1	7	0
8	0	62	1
16	3	139	5
32	3	151	7

[1] Minutes

[2] Tissues were paralyzed with magnesium, then treated as indicated. The results are expressed as percent of control twitch before addition of magnesium.

E. Further studies with dendrotoxin

Background

An effort has been made to clarify the mechanism by which drugs that alter potassium channel conductance can act as botulinum neurotoxin antagonists. This work has two motives. Firstly, there has been an assumption that the various serotypes of botulinum neurotoxin have essentially the same mechanisms of action. However, this assumption has been challenged by a variety of experimental findings, including those on 4-aminopyridine (4-AP) and its analogs. These drugs act on potassium channels to increase conductance, secondarily promoting influx of calcium and efflux of acetylcholine. 4-AP is a very effective antagonist of botulinum neurotoxin type A, but it is only weakly active or inactive against the other serotypes. Therefore, one motive for the work has been to determine why only one of the serotypes is strongly antagonized.

A second motive pertains to therapeutics. If one could determine the relationship between 4-AP and type A toxin, that could serve to point the way toward identifying drugs that would have similar relationships to the other serotypes.

Results

4-AP is regarded as a broad spectrum inactivator or potassium channels. There are other drugs that act more narrowly. The goal of this work was to identify a drug that acted on potassium channels, that promoted calcium influx

and acetylcholine efflux, but which did not act as a botulinum neurotoxin type A antagonist. This would allow for a kind of pharmacologic algebra. The channels affected by 4-AP minus the channels affected by the drug that is not an antagonist would include a pool of channels that are of importance.

A drug has been identified that satisfies the criteria above. In the initial round of experiments, the venom of Dendroaspis augustepsis was tested for its actions on neuromuscular transmission. In agreement with previously published findings by others, the principal investigator found that the venom has a dose dependent action. At low concentrations (~ 1 μg/ml) the venom facilitated transmission. This was manifested by a slowly increasing elevation in the muscle twitch amplitude of nerve-evoked responses (phrenic nerve-hemidiaphragm preparation). As the concentration was increased, so was the magnitude of the enhanced response and the rate at which the effect occurred. At its peak, the muscle response was enhanced about two-fold. With further increases in venom, there was still an enhanced response, but it was not sustained. Instead, the response waned and eventually the neuromuscular preparation failed.

The venom is known to contain a number of neurotoxins that are referred to generically as dentrotoxins. These toxins are the presumed agents mediating the facilitatory actions of the whole venom. Through the assistance of a

collaborator (Dr. R. Sorensen), one of dendrotoxins (I) was isolated and purified to homogeneity. This substance was tested on the isolated neuromuscular junction, and it produced the same spectrum of results as the crude venom.

Dendrotoxin as well as the whole venom were tested for their abilities to antagonize botulinum neurotoxin type A. Individual tissues were tit: ed with toxin or venom to produce a 50% to 100% increase in response. Botulinum neurotoxin type A (1×10^{-11} M) was then added, and the rate of onset of paralysis was monitored. The results indicated that neither the isolated dendrotoxin nor the whole venom possessed the ability to antagonize botulinum neurotoxin type A. The paralysis times of control tissues and pretreated tissues were essentially identical.

It has been found that even among those drugs that antagonize type A toxin, the effectiveness varies and tends to be highly calcium dependent. Therefore, experiments similar to those above were re-done, but in the presence of elevated calcium (3.6 mM and 7.2mM). The results did not change. Even in the presence of elevated calcium, neither dendrotoxin nor the whole venom significantly delayed the onset of botulinum neurotoxin type A-induced paralysis.

Dendrotoxin appears to satisfy the criteria discussed earlier. It inactivates potassium channels, it promotes calcium influx and acetylcholine efflux, but it does not antagonize botulinum neurotoxin type A. This means that the channels altered by dendrotoxin must not be the ones through

which 4-AP exerts its protective effect. Obviously it would be desirable to identify a drug that acts selectively on the relevant channels.

D. Studies on rubidium flux

Background

As explained in previous sections, there has been a convergence of interest directed at potassium channels in nerve cells and especially in nerve endings. There are at least three reasons for this, two of which are important to work conducted under this and an associated contract. To begin with, there are neurotoxin components from various venoms that exert their effects by virtue of interacting with potassium channels. An excellent example of this is dendrotoxin. A second reason, and one of importance to the contract work, is that at least two presynaptically acting neurotoxins are believed to bind wholly or in part to potassium channels. These are beta-bungarotoxin and crotoxin. And finally, a potent antagonist of one on the serotypes of botulinum neurotoxin (type A) is a broadspectrum potassium channel blocker (4-Aminopyridine and its analogues).

These various findings rightly focus attention on the potassium channel, but it must also be noted that the situation appears to be quite complex. This is due to the non-homogeneity of potassium channels, and it is also due to a series of unexplained and apparently anomalous findings.

A consideration of both is essential to the ongoing research.

Work on potassium channels has now revealed that there are at least four major types of ion flow that can be identified, and to some extent these classes can be subdivided. The four major classes are: i.) resting flux of potassium, ii.) a voltage-dependent, rapid flux that is inactivated, iii.) a voltage-dependent, slower flux that is not inactivated, and iv.) a calcium-dependent flux. In at least one case there is a strong interdependence. The voltage-dependent, rapid flux of potassium leads secondarily to opening of calcium channels. Calcium that reaches the cytosol then triggers the so-called calcium-dependent potassium flux.

An element of complexity enters the picture because the major classes of ion channels can be further subdivided. For example, slow potassium flux is very probably composed of at least two components. As another example, the channels that mediate a particular type of flux in one cell (e.g., voltage-dependent, rapid inactivating flux) may not be identical to the channels that mediate this type of flux in another cell. The common assumption among molecular biologists studying these channels is that they have all descended from a common ancestral gene, but there has been significant divergence with time. Also, the characteristics of individual potassium channels may be modified or even governed by the type of membrane in which they reside.

The complexities inherent in potassium channels are equaled by the seemingly anomalous findings that relate these channels to the actions of toxins that block exocytosis. This point was stressed during the last report, and the three most glaring anomalies were cited.

- 4-Aminopyridine and its analogues, by virtue of being potassium channel blockers, can antagonize botulinum neurotoxin type A, but they have much lesser or no ability to antagonize the other serotypes of botulinum toxin or tetanus toxin.

- Dendrotoxin, purportedly by virtue of being a potassium channel blocker, protects tissues against certain phospholipase A2 neurotoxins (e.g., beta-bungarotoxin), but it does not protect against other PLA2 neurotoxins (e.g., crotoxin).

- The data also reveal a crossed anomaly. 4-aminopyridine and its analogues can protect against one serotype of botulinum toxin, but it has not been shown to protect against any of the PLA2 neurotoxins; conversely, dendrotoxin protects against beta-bungarotoxin, but it affords no protection against any of the clostridial neurotoxins (last report).

It must now be reported that there is another unusual quality to the data on interactions. As just discussed, 4-aminopyridine and its analogues do not protect against PLA2 neurotoxins. However, they can potentiate the action

of these neurotoxins. As described below, the result is absolutely dependent on the rate of nerve stimulation.

The results obtained by the Principal Investigator and by others show that potassium channels are central to the study of presynaptic toxins. Unfortunately, it is unclear how channel function relates to toxin action. This is in part due to the absence of a methodology that is designed to characterize channels and to unravel the anomalies that have been discussed. Therefore, the past reporting period has been devoted to an effort to learn and master a new technique for studying potassium channels in situ.

Methods

Over a number of years Blaustein and his colleagues at the University of Maryland have developed techniques for studying the flow of ions across the membranes of isolated nerve endings. Their methods have involved the study of ions of interest (i.e., calcium), substitute ions that mimic those ordinarily associated with the action potential or with exocytosis (i.e., rubidium), and the monitoring of dyes that are indicators of cytoplasmic ion concentration (Blaustein and Goldring, 1975; Nachshen and Blaustein, 1982; Blaetschat and Blaustein, 1985). During the past Quarter investigators in this contract have collaborated with those in another to build an apparatus that would allow them to mimic the techniques used by Blaustein and his associates.

The procedure was to proceed through three steps. Initially a commercially available, small scale apparatus was purchased and modified. This apparatus was used in preliminary experiments to determine whether ion flux could be measured that was comparable to that previously reported. Next, a protein toxin was tested on the small scale apparatus, again to ensure that previously reported results could be obtained. Dendrotoxin was used as the test poison, as described by Benishin et al. (1988). Finally, an apparatus for large scale studies was designed and built at Jefferson.

The majority of the reporting period was devoted to building and testing the apparatus for studying ion flux. However, two other projects simultaneously went forward: i.) the study of toxins and channel blockers on phrenic nerve-hemidiaphragm preparations, and ii.) the establishment of a joint, international project (Madison, WI; London, GB; and Philadelphia, PA) to resolve a disputed point in the literature (see below).

Results

Rubidium Flux Experiments

The initial work with the commercially available apparatus and with dendrotoxin went well. Therefore, the results summarized here will deal with the apparatus that was built at Jefferson and with the data obtained using it.

The apparatus possesses 24 wells (3x8), each of which is capable of holding working volumes of 10 μl to 1500 μl. The apparatus can be used with any whole cell or re-sealed cell (e.g., synaptosome) preparation.

The essence of the procedure is that cells are preloaded with the isotope or dye of interest. In the present case, ^{86}Rb has been used as a marker for potassium flux. After being loaded, the cells are placed in the chambers of the apparatus and washed by filtration to remove unbound ion. The cells are retained by filters at the base of the top plate; the effluent can be directed either into collection vials or into a dump tube.

Typical experiments are conducted over an interval of 60 seconds. In the absence of calcium or depolarizing amounts of potassium, one can monitor resting efflux. The existence of calcium-dependent potassium flux is measured by the difference in efflux in depolarizing medium with calcium and the same medium without calcium. the distinction between the rapid, inactivating flux and the slow, non-inactivating flux is determined graphically by measuring ion flux over time; rapid flux inactivates within less than 10 seconds, but the slow flux continues throughout the experiment.

Our results have shown that all four times of flux can be monitored with ^{86}Rb (rat brain synaptosomes) In quantitative terms, the relative amounts of flux for the four components were:

Resting	~17%
Calcium-dependent	~20%
Rapid, inactivating	~23%
Slow, non-inactivating	~40%

Dendrotoxin was examined for its effects on flux in synaptosomal preparations. Within the concentration range of 10 to 1000 nM, it acted preferentially on the rapid, inactivation flux. This is in keeping with previous work done by electrophysiologists.

Aminopyridines and PLA2 neurotoxins

Data were provided that show that 4-AP does not antagonize the onset of neuromuscular blockade caused by beta-bungarotoxin. This finding would appear to be at odds with data reported by Chang and Su (1980). These authors did not find protection, but they did report a notable potentiation. When tested in the range of 10^{-5} to 10^{-4} M, 4-aminopyridine enhanced the rate of onset of beta-bungarotoxin-induced neuromuscular blockade.

Chang and Su (1980) and the present investigator have used similar concentrations of aminopyridine. However, the two laboratories have employed at least three differing techniques: physiological salt solution, rate of nerve stimulation, and concentration of beta-bungarotoxin. The salt solution was thought least likely to contribute to the dissimilar results. Therefore, toxin concentration and rate of nerve stimulation were varied. The results (Table 4)

show that toxin concentration was a minor contributor; the rate of nerve stimulation was the major factor.

It would appear that Chang and Su (1980) and the author are both correct. Aminopyridines do not protect against beta-bungarotoxin, but they can produce potentiation. The latter is a nerve activity-dependent phenomenon.

Table 4

Latin-square evaluation of toxin concentration and rate of nerve stimulation

	0.1 Hz	1.0 Hz
Beta-bungarotoxin (1×10^{-7} M)	220±19	191±16
Beta-bungarotoxin (1×10^{-6} M)	122±9	108±6
Beta-bungarotoxin (1×10^{-7} M) + 4-AP (50 μM)	174±16	185±14
Beta-bungarotoxin (1×10^{-6} M) + 4-AP (50 μM)	95±5	101±7

The data represent the mean±SEM of five preparations. The values are expressed in minutes.

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